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TUTORIAL

Fundamentals of Cancer Immunology and Their Application to Cancer Vaccines

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The capacity of the immune system to influence tumor progression has been a long-standing notion that first generated clinical traction over a 100 years ago when Dr. William Coley injected disaggregated bacterial components into sarcomas and noted that the ensuing inflammation commonly associated with tumor regression.[1] Since then, our understanding of the individual components and the overall interaction of the immune system has expanded exponentially. This has led to the development of a robust understanding of how components of innate and adaptive immunity recognize and respond to tumors and leveraging this information for the development of tumor immunotherapies. However, clinical failures have also deepened our knowledge of how tumors might adapt/be selected to avoid or inhibit immune responses, which, in turn, has led to the further iteration of immunotherapies. In this tutorial, the established elements of tumor immunity are explained, and areas where our knowledge base is too thin is highlighted. The principles of tumor immunity that guide the development of cancer vaccines are further illustrated, and potential considerations of how to integrate cancer vaccines with conventional therapies and other immunotherapies are proposed.

THE PREMISE OF TUMOR IMMUNITY

Numerous studies have shown that the presence of T lymphocytes within cancers is generally a good prognostic sign,[2–4] although the attention of a surgeon is still usually required to forestall tumor growth. The basic concept behind this prognostically advantageous event is that those T cells are recognizing something different about transformed cells, much akin to how they would recognize cells infected with a virus. This difference is commonly a change in the amino acid sequence of a normal protein that arises due to a mutation in the cancer cell's DNA (although other types of targets exist; see below). The T cell can peer inside the cancer cell because major histocompatibility molecules (MHCs; expressed by all nucleated cells) capture fragments of proteins (termed antigens) undergoing degradation within the cell and bring them to the cell surface. There they can be screened by T cells via their T cell receptor (TCR). If there is a fit, the T cell becomes reactivated; if not, it moves on by. Coding mutations give rise to neoantigens, or "new" antigens, and high affinity engagement of the TCR is referred to as signal 1 in T cell activation.

T cells (and B cells) are part of the adaptive immune response. It is termed adaptive because it is relatively flexible and durable, but relatively slow to initiate. In contrast, innate immunity is comparable to the eyes and ears of the immune system and serves as an alarm system. Innate immunity is ready to respond rapidly to "insults" and curtails infections for the short term, and initiates inflammation that supports and recruits adaptive immune responses. These mediators of inflammation are an important aspect of tumor immunology as they can provide both a biomarker of immunity in the tumor microenvironment (TME) and can be used as immunotherapies themselves. There are several critical elements to adaptive immunity that are particularly relevant to cancer immunology. First, it is exquisitely specific: the process of genetic recombination that develops B cell and T cell receptors leads to an extremely diverse repertoire that could respond to their corresponding ligands. It is estimated that there are $2.5x10^7$ potential combinations of TCR. This specificity empowers T cells to sense differences in peptides presented on the surface of cells, and also to sense poorly represented peptides (there is a lot of protein degradation occurring in each cell that provides the substrate for MHC molecules, and given that there are about 100,000 MHC molecules on each cell, there is a considerable amount of "noise" that the "signal" needs to be discriminated against on the surface of the cell). This specificity reduces the off-target effects that can occur with intensely activated, inherently nonspecific innate immune responses (consider toxic shock syndrome/sepsis that is a manifestation of cytokine storms from innate immune cells). Second, T and B cells remember. Memory responses, which arise after primary responses, are quicker and larger due to an increase in the proportion of T cell clones with relevant antigen specificity; metabolic and epigenetic changes that lower requirements for activation; and geographical localization; leaving them poised to re-expand upon re-encounter with their cognate antigen. This process of memory generation is a major goal of most vaccination programs.

There are two major classifications of T cells: cytotoxic T cells that express the CD8 coreceptor and respond to MHC class I molecules. These cells will release lytic granules that induce apoptosis in their target cells, and cytokines that can

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make cancer cells more visible and can promote inflammation in the TME; and helper T cells that express the CD4 coreceptor and respond to MHC class II molecules. The MHC class II molecules are more commonly expressed on antigen presenting cells (macrophages, dendritic cells, and B cells) in the TME, and CD4+ T cells produce cytokines that support effector activities of cytotoxic T cells, can turn macrophages into tumor killing cells,[5] and may make TME more accessible to immune cells. There is another type of CD4+ T cell that can be found in the tumor, termed a regulatory T cell (Treg), which will be discussed later. Thus, a coordinated cytotoxic and helper T cell response has the ability to recognize, respond, and eliminate cancers. If the response is sufficiently potent, it can eradicate the primary disease. The direct killing activity of cytotoxic T cells make them the obvious first choice for the development of cancer vaccines. Adjuvants are included in vaccines to try and mimic the support to cytotoxic T cells that would normally be provided by innate immunity. A greater understanding of interactions between T cell subsets supports the need for approaches that also include helper T cell activation.

B cells are the second arm of the adaptive immune response. Antibodies produced from B cells have clearly been useful in the diagnosis and treatment of cancer, but any direct role for B cells in the development of cancer, or contribution to either eradication or escape, has not been well-characterized. Thus, the remainder of this tutorial will primarily focus on T cells.

INITIATION OF THE T CELL RESPONSE

The term "reactivate" was used intentionally in the previous section. In order for T cells to perform tumor-controlling activities, they have to expand and differentiate. This is normally initiated in secondary lymphoid tissues, such as the lymph node. As each T cell has only one type of TCR, it generally has sufficient affinity to respond to only one MHC:peptide complex. Consequentially, we have a lot of different T cells with different TCRs. So, lymph nodes are designed to get T cells and their cognate antigen into the right place at the right time, rather than have T cells traffic through the blood stream until they randomly encounter their target. Naïve T cells express homing receptors that help them get from the blood into lymph nodes, where they await the opportunity to interact with antigen presenting cells carrying their cognate antigen. For the most part, antigen arrives in the lymph node having been captured in the periphery by dendritic cells (DCs). DCs exist in tissues particularly in regions that are likely to receive environmental exposure (lungs, skin, and digestive tract) and thus can sense the presence of invading pathogens. In the case of tumors, as tumor cells die (most commonly from deprivation of nutrients), they are engulfed by antigen presenting cells, such as DCs (**Figure 1**), using a recently described series of molecules to find dying cells and promote phagocytosis (e.g., CD47 blockade). In order to serve as more than mere vacuum cleaners, DCs must also sense that there is something wrong in the environment. In the case of infections, pathogen associated molecular patterns (PAMPs), such as single stranded RNA or lipopolysaccharide, activate pattern recognition receptors that are expressed by DCs and other cells, causing the release of cytokines that initiate an immune cascade. In the case of tumors, there are signals that are normally kept well within a cell: ATP, NAD, HMBG1, F-actin, and calreticulin, which are released by dying cells.

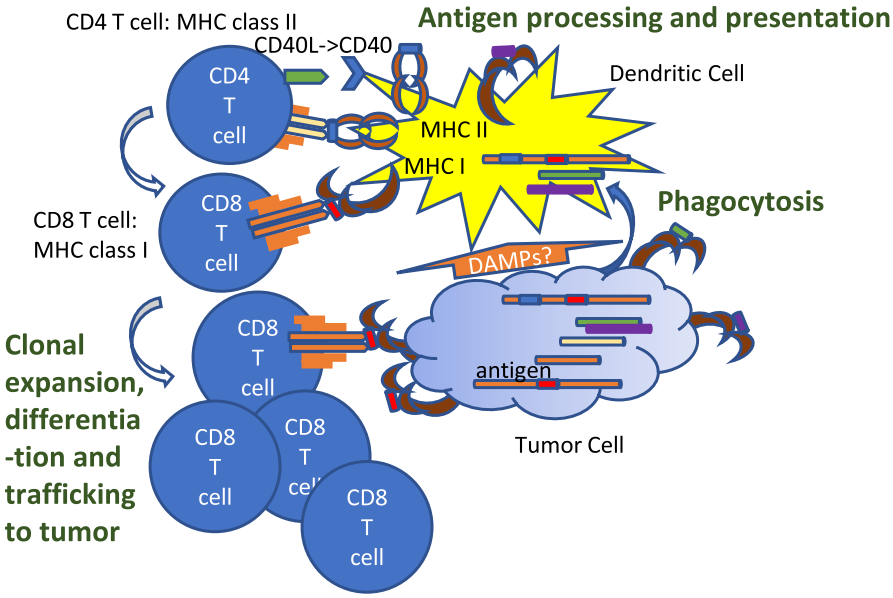

Figure 1 Dendritic cells (DCs) mediate the transfer of antigen and inflammatory context to T cells. DC acquire antigen from dying tumor cells by phagocytosis. Antigen is processed and presented on major histocompatibility molecule (MHC) class I and MHC class II molecules which engage CD8 and CD4 T cells, respectively. CD4 T cell activation can reciprocally stimulate DC by CD40L-CD40 interactions. Activated DC increase costimulatory molecule expression and promote the activation and expansion of CD8+ T cells. T cells undergo clonal expansion and depart the lymph node and traffic to the original site of inflammation. DAMPs, damage associated molecular patterns.

These signals are collectively known as damage associated molecular patterns (DAMPs) and can also stimulate DCs via pattern recognition receptors. These molecules serve as "find me, eat me, pay attention to me" signals that recruit and stimulate DCs. Interestingly, some chemotherapies and oncolytic viruses have been shown to promote the release of DAMPs from tumors as they die in a process that is termed immunogenic cell death due to its ability to enhance anti-tumor immunity.[6–8] In addition, natural killer cells, another subset of lymphocytes but without germline rearrangement and expression of antigen receptors, can be activated by stress-induced ligands expressed on tumor cells, and can recruit and activate DCs. Once stimulated, DCs stop phagocytosing material from their environment, and migrate through the lymphatics to the draining lymph node. They upregulate costimulatory molecules and MHC molecules that are important in T cell activation, and produce chemokines that attract T cells to them so that the naïve T cells can survey the MHC-peptide complexes on the surface of the DCs. Importantly, engaging the TCR (signal 1) alone is insufficient for full T cell activation (**Figure 2**). If signal 1 is received alone, T cells move into a state of unresponsiveness, termed anergy. To get full activation of T cells, they need a second signal (signal 2) that is provided by the co-stimulatory molecules on DCs and other activated antigen presenting cells (**Figure 2**). Costimulatory molecules, including CD80 and CD86 that bind CD28 on T cells, increase in expression according to the amount of the PAMP/DAMP signal. These costimulatory molecules can also be induced by helper T cells interacting with DCs via CD40-CD40 ligand, in a process termed licensing (**Figure 2**). Thus, in the case of slow growing tumors with little cell death, T cells are sometimes "ignorant" of their presence (i.e., there are few DCs in the tumor and they do not migrate with their antigen cargo to draining lymph nodes often) or there is insufficient DAMP stimuli to promote DC activation, resulting in T cell anergy.[9] This is the basis for treating tumors with viruses or PAMPs and why subunit cancer vaccines often incorporate PAMPs as one of their components. Further, as anergy is actually an active regulation of cell function, the signaling pathways and regulatory molecules that sustain this state of dysfunction are being targeted in the hope of reactivating these tumor-specific T cells. Note that anergy is considered a different state of dysfunction that is apparent in T cells that have been fully activated but then chronically exposed to antigen, a process that is referred to as exhaustion and characterized by the expression of multiple checkpoint inhibitory molecules on the surface of tumor infiltrating lymphocytes.[10] Checkpoint inhibitors can interfere with signals emanating from the TCR (PD1 recruits SHP and SH1P phosphatases) or compete for signal 2 (CTLA-4 binds more avidly than CD28 to CD80 and CD86). Thus, there are programmed constraints to activating a T cell response that must be overcome to promote the expansion, differentiation, and survival of T cellS responding to tumor antigens.

TRAFFICKING TO TUMORS

Once activated via TCR and costimulatory molecules, T cells undergo extensive changes driven by tyrosine and serine/threonine kinases and calcium release. These result in changes in T cell metabolism and transcriptional activity as they move from a state of quiescence to a state of effector activity. Substantial T cell division occurs, and T cells downregulate the homing receptors (e.g., CCR7 and CD62L) that are required for their presence within the lymph

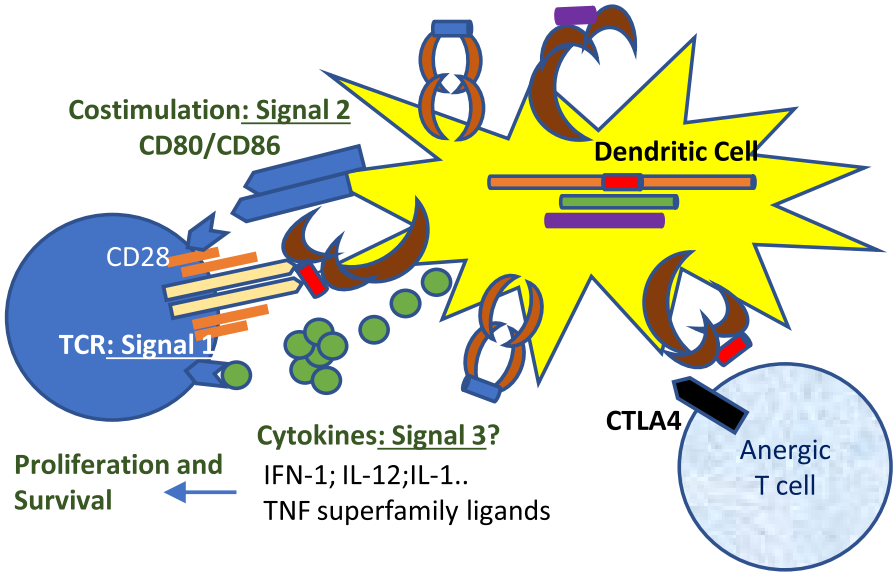

Figure 2 T cells need multiple signals to proliferate and differentiate. The primary signal is generated by T cell receptor (TCR) engagement of rare MHC:peptide complexes on antigen presenting cells (Signal 1). Activated antigen presenting cells co-express costimulatory molecules (CD80/CD86) engaging CD28 which amplify and sustain signaling pathways activated by TCR, leading to metabolic and epigenetic changes in the T cell (Signal 2). Cytokines and additional costimulatory molecules provide further support of proliferation, survival and differentiation into effector and memory T cell subsets (Signal 3). TCR signaling in the absence of Signal 2 leads to a state of functional anergy.

node, upregulate homing receptors (e.g., CXCR3, E-selectin ligand, and ICAM-1) that will provide them directions in the periphery, and move into the vasculature. Therein, they circulate until a combination of chemokines and integrins (**Figure 3**) at the target site cause them to start rolling on endothelial cell surfaces before adhering and then crossing across the endothelial cell lining.[11] This process requires sufficient inflammation in the TME to induce the expression of the homing receptor ligands for T cells (and other hematopoietic cells) to use as an address to exit. This targeting mechanism is considered to be one of the weaknesses of T cell responses induced by powerful vaccines (the T cells can traffic back to the vaccination site due to the inflammation present there) and cellular therapies, such as CAR-T in solid tumors.

RESISTANCE MECHANISMS

I refer to this aspect of tumor immunology as Newtonian Immunology: his third law of motion (approximately) states that for every action there is an equal and opposite reaction. In immunity, the stronger the immune response, the more mechanisms are activated to quell that response. An uncontrolled, unleashed immune response is actually startlingly dangerous to the host (witness the immune-related adverse events that can occur with cancer immunotherapies, especially combination checkpoint blockade) and, therefore, counter-measures to limit immunity are numerous. Unfortunately, in many instances, these immune-modulating activities are induced in the TME. For instance, cytokines, such as type-1 and type-2 interferons, that are produced by innate and adaptive immune cells within the TME will lead to the expression of molecules, such as programmed death ligand 1 (PDL1), indolamine dioxygenase,[12] vascular-endothelial cell growth factor[13–15] that dampen effector immune activity with a variety of mechanisms (**Figure 4**). Tregs, which play such a critical role in preventing autoimmune disease, are commonly recruited to the TME or the tumor draining lymph nodes.[16,17] Their propensity for preventing effector T cell responses from getting off the ground, or shutting down effector T cell responses once established, indicates that this cellular subpopulation needs to be carefully enumerated when characterizing a patient's tumor and peripheral blood. Failure to resolve the growing tumor has led to tumors being recast as "wounds that do not heal."[18] Aligned with this concept is the understanding that many of the immunosuppressive characteristics of a tumor can be related to a wound-healing response: molecular and cellular mechanisms of reducing inflammation (e.g., recruitment of type 2 macrophages and immature monocytes and the production of IL-10 and TGFβ) [19]; recruitment of fibroblasts; and endothelial cell growth and vascular development in response to nutrient deprivation and hypoxia. Add into this mixture the ability of tumors to compete for nutrients, such as glucose and amino acids,[20] the inherent hypoxia,[21] then it is easy to appreciate that the TME is not an ideal environment for effective antitumor immunity (**Figure 4**). However, this realization and understanding has led to strategies that either combat these resistance mechanisms individually (e.g., anti-PD1) or collectively by remodeling the cellular players in the TME (e.g., intratumoral injection of PAMPs or oncolytic viruses), or by strategies that "wipe the slate clean" in the TME (e.g., irradiation and chemotherapies). Although some of these strategies have clearly been successful, and in some instances durable, little is known about secondary

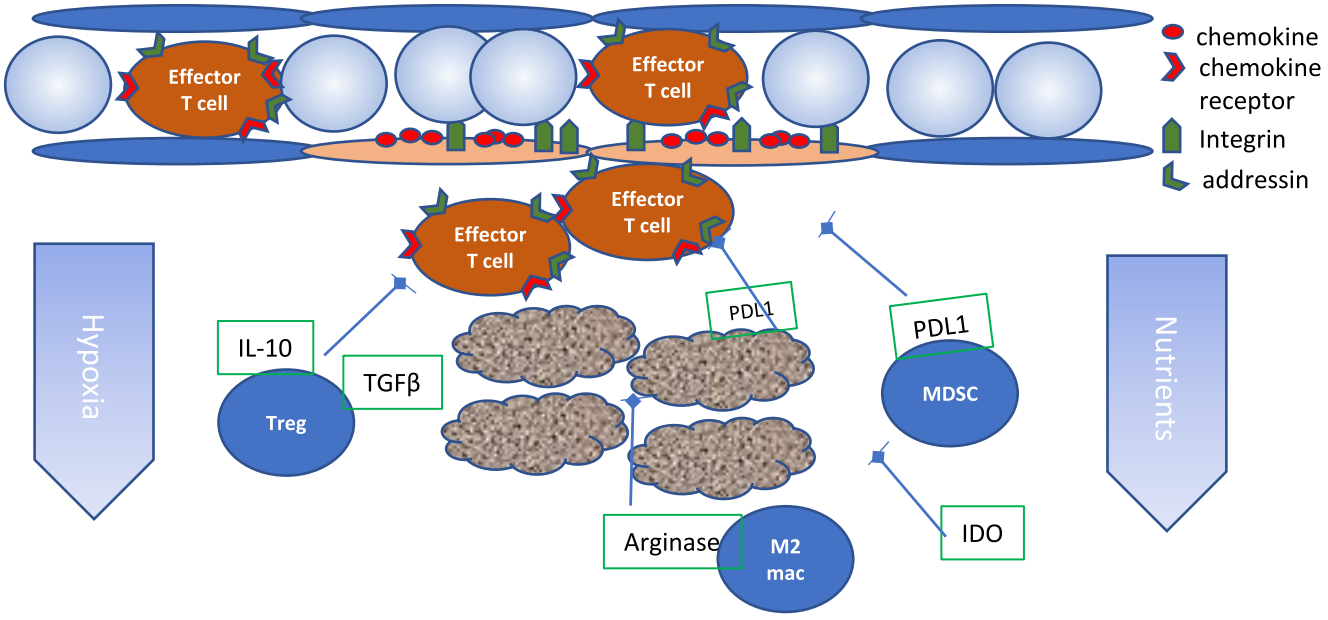

Figure 3 Activated T cells use chemokines and integrins as homing receptors to guide them to tumors. After leaving the lymph node, activated effector T cells upregulate chemokine receptors and integrins. These homing receptors are involved in rolling, arrest, and extravasation through endothelial cells lining the vasculature. Inflammatory cytokines in the tumor microenvironment (TME) promote the expression of chemokines and integrins on the tumor vasculature. Once in the TME, effector T cells can encounter many tumor and stromal barriers to function, and a metabolic environment that limits activity. Treg, regulatory T cell.

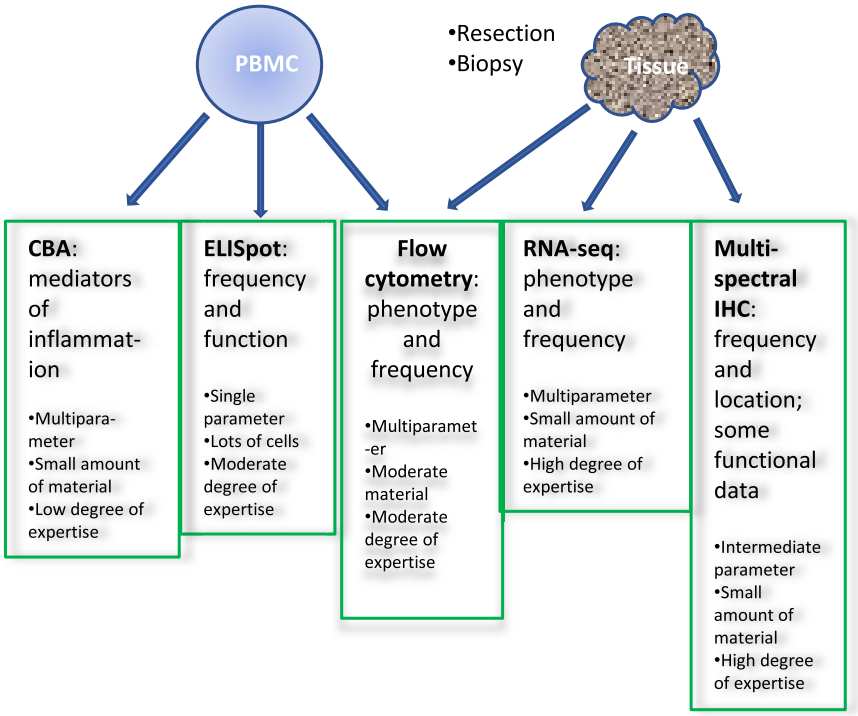

Figure 4 Standard assays used to assess quality and quantity of immune responses. Immune correlates studies use an array of assays that detect molecules or cellular populations and can characterize their phenotype and function at the single cell level. Types of assays chosen are predicated on the quality and quantity of material available. IHC, immunohistochemical; PBMC, peripheral blood mononuclear cell.

resistance mechanisms that occur as a function of tackling a primary resistance mechanism. Is there compensation by other resistance mechanisms? Does the primary resistance mechanism increase in intensity to overcome intervention? These questions will only be unraveled by the use of murine models that faithfully replicate the genetic and stromal elements of human disease, and the acquisition of on-trial biopsies. Added to these challenges, the immune system is an incredibly powerful selection agent, and it is becoming clear that a major mechanism that tumors use for hiding from T cells is by downregulation of MHC class I molecules.[22,23] Strategies are being developed to combat this (epigenetic modifiers; targeting natural killer and CD4+ T cell activation; CAR-T; bispecific antibodies),[24,25] but this loss of target availability is a major challenge for the success of cancer immunotherapies,[26] and is a fundamental example of the principles of the relationship between the tumor and the immune system as one of eradication, equilibrium, and escape.[27]

CHARACTERISTICS OF TUMORS THAT ARE CONSIDERATIONS FOR SELECTING IMMUNOTHERAPIES

Due to their durable efficacy and being relatively well-tolerated, selection of immune-based therapies as early line treatments is becoming more common. There is now a tremendous diversity of immune therapy types (antibody-mediated; cellular therapies; oncolytic viruses; and vaccines (discussed further below), but the most successful have been checkpoint blockade antibodies that are US Food and Drug Administration (FDA) approved in a variety of solid tumors, whereas checkpoint blockade and cellular therapies (including CAR-T) have been approved for the treatment of hematological malignancies. Bone fide prognostic indicators have been difficult to pin down for checkpoint inhibitors. Naturally, expression of the inhibitory ligand and the presence of T cells within tumors should be logical predictors of response.[28–30] Similarly, expression of the target for CAR-T is a logical entrance requirement for treatment. However, uncertainty over the proportion of tumor or myeloid cells that express ligands, such as PDL1 have clouded the utility of this marker. Similarly, the regional location of T cells within tumors, the density of T cells, and the neoantigen density (expressed as tumor mutational burden) have all been proposed as predictors of outcome.[31] However, part of the challenge in treating metastatic disease is the uncertainty over whether each metastatic site has unique characteristics. Further, much biopsy work is performed using relatively narrow-gauge cores and some studies suggest that several cores need to be examined to get an accurate assessment of the immune landscape.[32] Finally, an old story is re-emerging. The selection of tumors that had lost MHC class I expression, either to deletion of the class I region or due to loss of other molecules that are essential to MHC class I antigen presentation, was recognized as an immune escape mechanism decades ago.[33] However, as more patients receive checkpoint inhibition, the frequency and penetrance of MHC class I loss in solid malignancies is being appreciated with renewed interest.[34]

Of course, assessing all these prognostic indicators and mechanisms of adaptive resistance raise challenging questions of which parameters to focus on for a clinical trial, particularly if scarce patient-derived biopsies are the primary source of materials for both diagnostic and prognostic work up. This is particularly true for the analysis of on-trial biopsies. In some instances, allied use of RNA sequencing of the TME along with selective immunohistochemistry can provide a reasonably extensive understanding of the immune landscape and tumor diversity both prior to trial initiation and during the trial's progress. In other instances, multicolor or multispectral immunohistochemistry can help extract maximum information from limited materials.

A ROLE FOR CANCER VACCINES IN THE FUTURE IMMUNOTHERAPY REPERTOIRE

The array of immunotherapeutic approaches that can be applied for tumor therapy is ever expanding. Although it is clear that broad-spectrum use of checkpoint inhibitor antibodies has been relatively successful, their efficacy is significantly reduced in tumors that have limited T cell infiltration. Under these circumstances, ways to promote the presence of tumor-specific T cells must be considered. Cancer vaccines provide two unique opportunities with respect to the immunotherapy armamentarium. First, the intent is to generate a large number of tumor-specific T cells from sites distinct from the tumor. Spontaneously developed tumor-infiltrating lymphocytes have recently been characterized by single cell RNA-sequencing and flow cytometry as a heterogeneous population that can show characteristics of exhausted or effector-memory subsets,[35,36] with only the latter being able to respond to checkpoint inhibition. Interestingly, cancer vaccines also expand the more functional tumor infiltrating T cells in murine models.[36] Although immune responses to conventional vaccines against pathogenic infections have been quite well-characterized,[37,38] and relatively broad immune characterization of cancer vaccines indicate that effector-memory subsets are commonly generated in the periphery. These recently expanded T cells will have been activated under relatively enhanced conditions, limiting anergy, and will have had little opportunity for the tumor microenvironment, if present, to restrain their activity by exhaustion or other immunomodulating techniques. These characteristics suggest that there is a greater likelihood of developing long-lived memory T cells that will be numerically and functionally poised to respond to re-emerging metastatic disease after vaccination compared with those that linger after tumor resection. For these reasons, cancer vaccines have long been touted as a potentially effective way of treating emergent tumors. The concept of cancer vaccines has existed for several decades, because the initial tumor antigens were identified (see below), but has consistently underproduced with respect to efficacy in patients, at least in the therapeutic setting.[39] There are several major questions that face "vaccineers" as they develop novel approaches for expanding tumor-specific T cells that show evidence of efficacy at controlling disease advancement or recurrence:

Targets

T cells recognize a remarkably wide array of tumor antigens, each of which have advantages and disadvantages (**Table 1**). The earliest efforts to identify the targets of T cells within tumors used two competing methods. On the one hand, groups generated cDNA expression libraries from tumor transcripts, and screened pools of cDNA for T cell reactivity by transfecting MHC-matched target cells. Where reactivity was observed, the pool was subdivided until an individual transcript could induce T cell responses. The transcript was sequenced and the target gene subsequently identified. Many of the cancer-testis-differentiation (CTA) antigens were defined in this manner.[40–42] An alternative approach, in which peptides were eluted from the MHC molecules expressed by cancer cells, and then screened in pools by examining T cell reactivity after pulsing onto antigen presenting cells.[43,44] Many tumor-associated antigens that are commonly expressed by normal tissues were identified by this technique. More recently, the power of whole exome sequencing and RNA sequencing, aligned with improvements in algorithms that predict MHC binding sites and proteolysis by enzymes involved in antigen processing, has led to a surge in the identification of antigens that are specific to an individual patient's tumor.[45,46] There is a healthy debate as to the best class of antigen to include in vaccine development. Shared antigens have the advantage

Table 1 Tumor antigens exist in many forms, with varied penetrance in expression with tumors and normal cells

Type of tumor antigen	Example	Strengths	Weaknesses	Tumor type
Tissue differentiation	Tyrosinase; Surface Ig	Commonly expressed	Tolerance	Melanoma; lymphoma
Cancer-testes	MAGE; NY-ESO; CEA	Low tolerance; commonly expressed	Variegated expression	Melanoma; breast cancer; colon cancer
Abnormal post-translational modification	Muc-1; Phosphopeptides	Common expression; driver of transformation	Some expression in normal tissue	Breast cancer; pancreatic cancer
Oncoviral protein	HPV E6, E7; hERV	Foreign		Cervical
Mutated oncogene	CDK4; KRAS; BRAF	Driver of transformation	Limited MHC restriction	Melanoma; lung cancer
Neoantigen		No tolerance	Unique to each tumor	various

HPV, human papillomavirus; MHC, major histocompatibility molecule.
These variations influence the quality and quantity of any T cell response and the extent to which tumors might be edited to lose antigen expression in response to immune recognition.

of being commonly expressed within a tumor type (for example, tyrosinase in melanoma), making vaccine formulation more universal. However, they may be subject to self-tolerance leading to a smaller repertoire of T cells to respond. T cell repertoires specific for CTA antigens are less likely to have undergone tolerance-driven pruning as CTA are not expressed in the thymus, and CTA are expressed by many tumor types. However, their expression is commonly in a mosaic pattern, with not all tumor cells expressing CTA equivalently, given rise to the concern that escape variants will be rapidly selected. The advances in DNA and RNA sequencing have resulted in an appreciation of the array of potential targets for T cells in the form of mutations that arise to alterations to amino sequences. When aligned with algorithms that predict binding to MHC molecules, neoantigens can be identified and synthesized into vaccines, whether they be peptide or RNA-based.[47–52] Neoantigens are likely highly immunogenic, yet they are the epitome of personalized medicine and thus are relatively expensive to make. It remains a concern that for each of these subtypes that, unless the targeted antigen is essential for the cancer's transformed state, selection of loss variants will occur. Thus, some vaccines are targeting proteins that are essential for oncogenesis (e.g., KRAS, BRAF, and E7) and others the intermediate substrates (e.g., phosphopeptides).

Formulation

Antigen. Tumor antigens can be delivered in multiple formats, each of which has strengths and weaknesses. The most direct is via protein or peptides that are the direct mimic of the target expressed by the tumor cell. Advantages here are that most post-translational modifications can be incorporated into the antigen during synthesis if produced in animal cells. Yet, synthesis is costly and relatively slow. Peptide half-life in serum is relatively low compared with proteins. Recombinant proteins are a major avenue of development, including those connected to targeting antibodies. mRNA encoding the tumor antigen can be rapidly synthesized, but efficiency of transfection remains low particularly in professional antigen presenting cells. Thus, mRNA is often delivered in vectors that are either encapsulated in targeted liposomes or incorporated into viral vectors, such as attenuated ALVAC[41] or adeno-associated viruses (AAVs).[53] DNA vaccines expressing the coding sequences of the targeted antigen are a further option, and ways of more effectively delivering nucleic acids is a major focus of many nanotechnology laboratories.[54]

Adjuvant. Adjuvants provide an inflammatory context for antigen presentation and help prevent the induction of T cell anergy. One reason for the relatively low level of immune responses obtained with peptide/protein/nucleotide cancer vaccines to date, as compared with the magnitude of response to viral infections, is likely related to the choice of adjuvant. Historically, the primary adjuvant used for the delivery of peptide and protein vaccines has been the "oil-in-water" emulsion approach (incomplete Freund's adjuvant; e.g., Montanide), or alum.[55] Both of these adjuvants were primarily developed for promoting antibody responses. Although it is acknowledged that strong CD4+ T cell responses are needed to support B cell responses against T-dependent antigens, this is likely not an optimal approach for promoting cytotoxic T cells. Various additional components, including cytokines, intending to promote DC recruitment (e.g., GM-CSF) and TLR agonists (e.g., polyICLC) or recently defined activators of innate immunity (e.g., cyclic guanosine monophosphate–adenosine monophosphate have been tested as monotherapies or in conjunction with first-generation adjuvants, such Montanide and alum, in order to increase cellular immune responses.[56] Recently, concern has been raised that these traditional vaccination approaches may actually serve as an inflammatory decoy for activated T cells, luring them from the less inflammatory tumor.[57–59] This has led to the consideration of other adjuvants and delivery systems for cancer vaccines. The most obvious are DCs themselves.[60] Recombinant viruses have also been extensively developed,[61] as they bring their own natural adjuvants (generally via TLR receptors and type 1 interferon responses). However, there are inherent challenges with dosing virus-based vaccines so that viral burden and toxicities are tolerable in patients with cancer, and the barrier of potential pre-existing immunity to many of the viral backbones that are currently deemed safe in humans. Exogenous cytokines, including IFN-1, IL-12, and IL-27, have also been considered, but are constrained by getting sufficient local activity without driving systemic toxicity. To address this, a multitude of nanoparticle formulations are under preclinical testing,[62] with many of these based on liposome scaffolds or particular aggregates. Both of these "vectors" can be delivered either passively (relying on the enhanced permeability and retention effect) or by active targeting with antibodies, ligands, or aptamers, in particular, targeting receptors on DCs, such as DEC-205, Clec9a/DNGR, and the mannose receptor. Encapsulation can reduce some of the toxicities associated with these adjuvants and increase the selective targeting to either the tumor site, or lymph nodes where T cell priming should occur. Although full of potential, nanoparticle vaccines are also limited due to either rapid clearance and systemic toxicity or limitations on payloads they can carry or the amount that can be delivered. Thus, much work has been performed on understanding the steps needed for construction of strong CD8+ T cell responses. Clearly, the activation of DCs is a primary focus, and thus nucleotide TLR agonists such as CpG and polyICLC have been investigated, along with TLR agonists composed of bacterial cell wall molecules (lipopolysaccharide and flagellin), but a balance between achieving sufficient transient local delivery that can prime a substantial T cell response and concerns over systemic toxicities remain difficult to achieve. An interesting avenue has been the use of cross-linking antibodies to promote or mimic the engagement of TNF-superfamily receptors expressed by T cells. These molecules include CD40, CD27, OX40, 4-1BB, and GITR. These antibodies have the ability to substantially expand T cell responses to cancer vaccines in many forms, leading to high circulating levels of tumor-specific T cells in preclinical models.[63] Again, some of these antibodies (CD40 and 41BB) have caused adverse events and need to be carefully titrated,[64,65] whereas others are more well-tolerated in patients and are currently

being explored in clinical trials either as monotherapies or in combination with checkpoint inhibitors and other modulators of the TME. Going forward, it will be important to understand what biomarkers are available to indicate favorable adjuvant qualities (circulating cytokine levels and RNA in exosomes, for example). Additional considerations are the targeting of adjuvants to tumor sites in order to support and sustain the expanded T cells as they enter the TME. Further, as the inflammation and qualitative aspects of adjuvants are perceived by T cells responding to cancer vaccines, there remains the potential to develop vaccine composition such that that it will promote a desired T cell differentiation state and the tissue sites that they localize to. These fundamental properties of immunization strategies are akin to those found in infectious diseases (the generation of memory cells that can rapidly respond to re-exposure to the pathogen (e.g., influenza) or to achieve sterile immunity against a chronically infecting pathogen (e.g., HIV and cytomegalovirus). They are also important when considering which patient populations are best for assessing the efficacy of a vaccine. If the vaccine is intending to induce robust antitumor immunity that prevents recurrent disease via promoting memory T cells, assessment would be best performed in patients resected and restaged to no-evidence of disease. Alternatively, if the vaccine is intended to generate large numbers of short-lived effector cells, which may not develop robust memory, then patients with existent metastatic burden may provide the best cohorts for studying the efficacy of the vaccine. Finally, issues of guiding responding T cells to different anatomic sites remain, in particular access to the central nervous system to engage metastatic disease, has not been extensively evaluated.

Measurement of immunity and the response to vaccines

The previous section has illustrated that there is an enormous number of potential combinations of antigens and adjuvants that could be integrated into cancer vaccine design to be deployed in the clinical setting. Clearly a major parameter of a successful vaccine is the capacity to measure the magnitude and functional activity of the responding T cell population, preferably within the TME. As the majority of cancer vaccines target the expansion of CD8+ and CD4+ T cells, and in many cases the immunizing antigen is available for *in vitro* assays, ELISpot cytokine release assays are most frequently used to assess the magnitude of the response due to their sensitivity, specificity, and relatively high throughput.[66] As they use the patient's peripheral blood mononuclear cell (PBMC) as the antigen presenting cells, having the appropriate MHC restriction is not an issue, although a battery of antigen presenting cells transfected to express particular MHC molecules is available. ELISpot assays are performed either directly *ex vivo* from patient PBMC, or after a short *in vitro* stimulation with the immunizing antigen. The majority of ELISpot assays use IFNγ as the reporting cytokine, although there is interest in pursuing other reporters of function to interrogate the polyfunctional nature of the responding T cell populations. Generally, ELISpot assays require relatively large numbers of T cells and so are most commonly performed on PBMCs, leaving open the question of whether the induced immune response is reaching the tumor sites and is active there. The second major approach for measuring immune responses to vaccines is via flow cytometry. These incorporate dye-dilution (e.g., carboxyfluorescein succinimidyl ester) assays, MHC tetramer-staining (physically identifying vaccine-specific T cells), or combinations of activation markers, such as HLA-DR and CD38 to identify vaccine-responsive T cells. Assays are performed either directly on isolated PBMCs or after PBMCs are restimulated *in vitro* with the antigen backbone of the vaccine. The power of flow cytometry is that relatively few cells are needed, thus allowing the interrogation of tumor biopsies or resections, and the variety of surface and intracellular molecules that can be used to phenotype the quantity and quality of the response.[67,68] Novel technologies, such as Mass Cytof and barcoding, have allowed the development of relatively high throughput and high-dimensional data acquisition that can be interrogated for diagnostic and prognostic biomarker development, and how changes in vaccine formulation influence these aspects of the T cell response to vaccination.[69,70] When combined with other single-cell technologies, such as RNA-sequencing and "window" trials that have a timeline of vaccination prior to resection, a very granular interrogation of the patients' T cell response can be generated, and can place the T cell response in the context of any changes that occur in the other immune cell components in the TME.[71] It should be noted that these high-dimensional techniques are currently used for hypothesis generation, rather than clinical evaluation. Importantly, analyzing TCR expression can directly determine whether T cells that are found in tumors in response to vaccination are the same clones as found in circulation, and this provides a readout for determining whether the vaccine has effectively recruited T cells into the TME methods to image immune response are also under development, with recent exciting progress using radiolabeled-diabodies (small recombinant forms of antibodies) allowing real-time positron emission tomography tracing of expanding T cell responses to vaccines, or other immune interventions.[72,73] Clinical trials will be needed to establish the sensitivity and precision of imaging approaches to help estimate intratumoral immunity. It should be noted that analysis of tumor tissue will be dependent upon how much material is available, and, in many instances, performing RNAseq in combination with algorithms such as CIBERSORT[74] can provide a good first step in understanding the immune context of the tissue, which can then be probed more precisely with immunohistochemical-based techniques.

Naturally, the overall ability of a vaccine to develop antitumor immunity should be considered in the context of the therapeutic effect of the vaccine. In the setting of a prophylactic vaccine, most commonly deployed after surgical reduction to no-evidence of disease, then time to recurrence is the most feasible measure. In patients with diseases that have a high likelihood of recurrence even after complete dissection (due to the seeding of metastases that are not radiologically detectable at time of surgery), efficacy can be measured as time to recurrence.[75,76] In the

context of therapeutic vaccines, radiological assessment of tumor size likely correlates with immune infiltration and effector activity. Response Evaluation Criteria in Solid Tumors (RECIST) and immunotherapy RECIST (iRECIST)[77,78] assessment of tumor progression and determination of overall survival will reflect on the efficacy of vaccines to control disseminated disease (**Figure 4**).

Immunocompetence

An additional aspect for consideration with respect to cancer vaccines is the patient's immune competence. Common sequelae of cancer are immunosuppressions, so having some gauge of immune competence is helpful in interpreting the potency of vaccine formulations. Minimally, the baseline lymphocyte counts in PBMC should be known. Preferably, a functional assessment in response to known stimuli, such as PHA or the CEF panel of peptides (that measures immunity to common pathogens cytomegalovirus, Epstein–Barr virus, and influenza) should be incorporated into the assessment of immunity. Understanding how resistance mechanisms within the TME are influenced by components of vaccines is also related to overall immunocompetence in the TME. Thus, do particular adjuvants promote the differentiation of immature myeloid cells to M1-type macrophages? Do viral vaccine delivery systems (especially intratumoral) promote the expression of checkpoint ligands, such as PDL1 or elaborate the expression of other immunosuppressive elements such as arginase?

Scheduling vaccine delivery

Aside from the composition of the vaccine, qualities of the vaccine regimen need to be considered. The vaccine can be altered by the amount of vaccine delivered in each injection, the delivery location, or the frequency of the injection. The impact of varying the frequency, location, and amount of vaccine can be quite different, from an immunological perspective, than from traditional pharmacodynamic/pharmacokinetic considerations. As discussed earlier, memory T cell responses generally expand more rapidly and to a higher degree than primary T cell responses, and this aspect of active immunity has been leveraged with prime-boost immunization approaches.[79–82] Interestingly, whether it is possible to achieve true memory cell differentiation (epigenetic and metabolic) when tumor remains in a patient is a subject of debate, and may influence the vaccine scheduling. Repetitive weekly vaccines may boost effector cell responses, but may limit memory cell development as compared with monthly or more widely interspersed immunizations. Commonly, cancer vaccines are initiated after surgery has debulked disease, but some evidence indicates that neo-adjuvant vaccination can lead to a survival benefit, perhaps due to a boosting effect from released tumor antigen and inflammation.[83] However, cytoreduction surgery is also thought to reduce the global immunosuppression associated with some tumors, making T cell responses to vaccines more effective. The composition of the vaccine can also influence the duration of both antigen and inflammation, and thus T cell exposure: peptides are rapidly proteolyzed in serum[84,85]; antibodies can adsorb viral particles; adjuvants can cause antigen retention and sustain inflammation at the injection site.[58] Many of the aspects that lead to the optimization of vaccine regimens are first established in murine models before being expanded to humans in early phase clinical trials.

Immune correlates and immune monitoring vaccine efficacy

Unfortunately, there are very few established prognostic biomarkers that have been harmonized across cancer vaccine studies that allow prediction of the quality of the immune response aside from directly and empirically assessing the frequency and function of the T cells responding to vaccines. Most initial studies are performed in animal models, with rodents predominating due to the relative equivalency between their immune responses and that which occurs in humans. An important caveat is that tumor models in mice are imperfect given that they are either implanted, or genetically induced with accelerated emergence and low mutational burden. Further careful selection of mouse models will allow assessment of the vaccine's efficacy against both primary disease and subsequent metastases.

In order to empirically study the effectiveness of cancer vaccines in humans, a well proscribed sampling timeline needs to be implemented. Clearly, baseline, pre-vaccination, responses are required, but additional considerations are dependent upon the frequency of the vaccination and the intent of the trial. Some vaccination strategies involve weekly injections whereas others are delivered at a lower density, likely with different goals (expanding effector T cells vs. generation of memory), dictated by the extent of disease, its location, and the degree of inflammation that occurs with immunization. Most protocols use additional assessments of T cell responses in blood drawn prior to the next vaccine delivery, with additional blood draws at the end of the vaccine regimen to understand the durability of the vaccine-driven response. This has ramifications with respect to the cost of the immune correlated studies and the ability to recruit patients, some who may not have the inclination or the means to travel for weekly blood draws. However, PBL can be shipped to a central monitoring laboratory with reasonable viability. Mathematical modeling of the activity of cancer vaccines is a relatively nascent stage,[86–88] but may provide a promising avenue to estimate the interactivity of the various components of cancer vaccines.

Impact of conventional therapies

In the current environment, immunotherapies are seldom delivered in a vacuum, and are often part of combinations that are intended to support immune activation by augmenting tumor damage, which could be considered a form of auto-vaccination, or by attenuating adaptive resistance mechanisms. Further, where established standards of care treatments are in place, it is important to understand how these allied interventions might influence the outcomes of a cancer vaccine.

Chemotherapy. The vast majority of chemotherapies are designed to eliminate rapidly dividing cells, which unfortunately is also a major characteristic of an activated immune response! *A priori*, one might expect chemotherapies to be

paradoxical to tumor immunity. However, the immunological ramifications of chemotherapies have been extended by recent studies indicating that subsets of chemotherapies can induce a form of cell death that releases DAMPs.[6,7,89,90] The increased immunogenicity of this type of cell death has primarily been demonstrated by the ability of the therapies to convert tumors into cancer vaccines (i.e., mice implanted with chemotherapy-treated tumors subsequently demonstrate protective immunity upon rechallenge with fresh tumor). Further, standard of care chemotherapy has been shown to cooperate with checkpoint inhibitor blockade in non-small cell lung cancer,[91] indicating that there is room for these therapies to work in tandem. Clearly, a tremendous amount of work with on-trial biopsies is needed to understand the basis of this cooperativity, and understanding the sequencing of chemotherapies with immunotherapy is critical. Notably, metronomic cyclophosphamide has also been shown to selectively deplete Tregs, but whether this can improve the effectiveness of cancer vaccines *in vivo* is less clear.[92] Certainly, it is worth considering whether the acute inflammation that is induced by chemotherapy-induced cell death would promote the recruitment of vaccine-induced T cells to the tumor site. Further, it is possible that chemotherapeutics can "wipe the slate clean" eliminating both failed effectors and regulatory populations within the TME to allow infiltration of *de novo* responses propagated by cancer vaccines.

Radiotherapy. A well commented aspect of radiotherapy is the abscopal effect, whereby sites beyond the original target of irradiation also respond to therapy. This is most simply understood as the generation of a systemic immune response by the radiotherapy, either by release of tumor antigens or disruption of tumor-mediated immunosuppressive networks.[93] However, substantial evidence of interactivity between irradiation and immunotherapy has not been forthcoming, and other studies have suggested that wide-field irradiation can cause immunosuppression, either by directly attenuating lymphocytes[94] or due to the release of immunosuppressive cytokines, such as TGFβ. [95] Interestingly, *in situ* vaccination approaches, wherein local DCs are activated by CD40 stimulation, have offered some benefit after stereotactic irradiation, suggesting room for advancing cooperation between radiation and cancer vaccines.[96]

Immunotherapy. The burgeoning understanding of the relationship between immunity and cancer has provided opportunities to manipulate systemic immunity in conjunction with vaccination. In some instances, the intent is to remove immunosuppressive elements that could either prevent or attenuate the development of the immune response elicited by the vaccine. Examples would be depletion of Tregs or immunosuppressive myeloid cells; blocking immunosuppressive cytokines or enzymes such as TGF$^{\beta}$[97] or ectonucleotidases (e.g., CD73)[98]; blocking checkpoint molecules (e.g., CTLA-4 or PD-1[99]). Conversely, other combinations of additions include boosting T cell immunity with antibodies that stimulate T cell coreceptors, such as 41BB (CD137)[100] or CD27.[101] It should be noted that although these further manipulations of the patient's immune system could result in enhanced antitumor immunity, releasing the inherent constraints of the immune response can lead to a variety of immune-related adverse events with various degrees of intensity, morbidity, and mortality.[102]

SUMMARY

The immunological cellular players, and many molecular mechanisms, that are engaged in either eradicating tumors or supporting their escape have been identified and characterized. As immunotherapies are implemented, we will learn how either pre-existing or *de novo* generated adaptations limit antitumor immunity. This, in turn, will lead to the development of biomarkers that will help select patients for particular immune-based interventions, or companion therapies that could attenuate resistance mechanisms. Implementing rigorous clinical trials and biopsy collection, aligned with innovative mouse models, and utilizing standard and state-of-the-art analytical approaches will allow both validation and extension of the efficacy and durability of cancer immunotherapies. However, basic preclinical research can be especially important to help understand and compare avenues of opportunity.

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